RESEARCH ARTICLE

High-yield and Functional IgY Anti-S1 SARS-CoV-2 Delta Variant from Chicken Immunized with In-house Recombinant Protein

Kamaluddin Zarkasie[1], Teridah Ernala Ginting[2,*], Andrea Hynan Poeloengan[3], Febi Andriani[2], Aksar Chair Lages[2], Vina Cornelia[2], Anggah Nugraha[1], Bustanur Rosidi[2], Setyarina Indrasari[4], Chairul Anwar Nidom[4,5], Irawan Yusuf[2]

[1]PT IPB Shigeta Animal Pharmaceuticals, Faculty of Veterinary Medicine, Bogor Agricultural University, Jl. Agatis, Bogor 16680, Indonesia
[2]Mochtar Riady Institute for Nanotechnology, Universitas Pelita Harapan Medical Science Group, Jl. Boulevard Jenderal Sudirman 1688, Tangerang 15811, Indonesia
[3]National Resilience Institute of Republic of Indonesia, Jl. Medan Merdeka Sel. No.10-11, Jakarta 10110, Indonesia
[4]Professor Nidom Foundation, Wisma Permai Tengah I, Surabaya 60115, Indonesia
[5]Faculty of Veterinary Medicine, Universitas Airlangga, Kampus C Mulyorejo, Surabaya 60115, Indonesia

*Corresponding author. Email: tginting@mrinstitute.org

Received date: May 19, 2025; Revised date: Jun 19, 2025; Accepted date: Jun 25, 2025

Abstract

BACKGROUND: During COVID-19 pandemic, effective preventive and therapeutic strategies was urgently necessary, especially with the emerging Delta variants. Previous existing interventions have some limitations, and passive immunization using immunoglobulin Y (IgY) is considered viable. However, the potential of IgY antibodies remains underexplored locally. Therefore, a feasible study was conducted to produce IgY against the SARS-CoV-2 Delta variant from chickens raised on a farm, instead of a laboratory environment, by using in-house recombinant protein, to offer a promising low-cost alternative for passive immunization.

METHODS: In-house HEK293 cell line was produced by secreting recombinant S1 subunit protein of SARS-CoV-2 Delta variant. The protein was confirmed by western blot and used as an immunogen in two chickens. IgY was extracted from egg yolks. Titers and neutralization activity of IgY against SARS-CoV-2 Delta variant were confirmed by enzyme-linked immunosorbent assay (ELISA) and surrogate virus neutralization test (sVNT). The functionality of IgY was then tested on lysates from COVID-19 patients' nasopharyngeal swabs with dot blot method.

RESULTS: The high anti-S1 IgY titers and neutralization activity was confirmed following immunization with 1 mg immunogen at 2–3-week intervals. IgY titers varied between chickens and time points. The mean titers showed significant increase after the fifth immunization (1.21, 1.72 and 1.48; with $p<0.05$ for all). Neutralization activity appeared after the second immunization and was significant after the third immunization (31.9%, 34.95%, 26.9%, 47.6%, 54.95% and 57.3%; with $p<0.05$ for all). The results of this study showed that the extracted IgY reacted to COVID-19 patients' nasopharyngeal swabs lysates.

CONCLUSION: A high-yield and functional IgY anti-S1 SARS-CoV-2 Delta variant can be produced from chicken immunized with an in-house recombinant protein. This can be an alternative for affordable and effective IgY production during public health emergencies.

KEYWORDS: IgY, SARS-CoV-2, S1 subunit protein, egg yolk, chicken

Indones Biomed J. 2025; 17(4): 363-72

Introduction

COVID-19, a disease caused by a novel coronavirus SARS-CoV-2, started emerging in December 2019 and caused over 6 million deaths by March 2022 hence become a global pandemic.(1–3) It is associated with symptoms that include fever, chills, fatigue, and respiratory, digestive, and cardiovascular issues.(4) Spike (S) protein of SARS-CoV-2, which become one of the vaccination targets, consists of S1 and S2 subunits. S1 subunit recognizes and binds to the host receptor angiotensin-converting enzyme 2 (ACE2), while S2 subunit modulates fusogenic activity to host cell.(5–8) It has been reported that substitutions at spike-protein positions 417, 452, 484, and 501 in S1 subunit contribute to antigenic shift.(9) Consequently, S1 subunit protein is a suitable immunogen to induce protective antibodies.(10–12) The first widely reported SARS-CoV-2 mutation was the substitution of aspartate with glycine at residue 614 (D614G) in 2020, followed by variants like Delta (B.1.617.2), which is highly transmissible, associated with higher viral loads, and linked to increased hospitalization and mortality.(13) By mid 2021, Delta variant had superseded the Alpha variant, accounting for over >90% of cases worldwide.(14)

Rapid and practical approaches are crucial in pandemic emergencies. Available treatments such as chloroquine (QC) and hydroxychloroquine (HCQ) were used early in the pandemic for COVID-19 treatment in Indonesia, although clinical trials showed no significant benefits.(15) While vaccination and antiviral agents help prevent infection, their development is slow and costly.(16,17) Passive immunization is considered a viable alternative.(18) Chicken immunoglobulin Y (IgY) is a homolog of human immunoglobulin G (IgG) that is found in avian serum and egg yolk. It is a cost-effective, high-yield, immunoglobulin source with a strong safety profile and minimal environmental impact and infrastructure investment.(19,20) The required immunogen concentration and antigen type to produce IgY vary by case, with multiple immunization protocols tested to maximize antibody titers. Studies showed hens generated strong immune responses to bovine serum albumin at doses of 0.1–1.0 µg, with higher doses (10–1000 µg, most often 50–100 µg) were also used.(21,22) IgY has also been produced using complex antigens (viruses, bacteria, parasites) and single antigens (proteins, polysaccharides, peptides, nucleic acids).(21) The use of IgY for passive immunization source has been evaluated *in vitro* and *in vivo* against SARS-CoV-2, influenza virus, Ebola, Zika, Dengue and Norovirus with promising results.(22–26) In pig model, systemic and local IgY administration provided protection against Rotavirus with minimal adverse effects and inflammation.(27,28) However, there is lack of local documentation on a prompt production of IgY during pandemic emergencies in a limited infrastructure and access to the sufficient amount of field isolates.

Indonesian government had initiated efforts to accelerate the management, prevention, control and mitigation through local research and invention. A quick and low-cost immunoglobulin against SARS-CoV-2 was one of the objectives. Although IgY production in chickens for other pathogens was well-documented, the country lacked of cultured SARS-CoV-2 Delta variant isolate for use as an immunogen. To address this, a recombinant Spike S1 protein subunit of the SARS-CoV-2 Delta variant was produced and used to immunize layer chickens raised for egg production. The resulting IgY's potency using ELISA, neutralization assay and reactivity test to clinical specimens was then evaluated. The results of this study support the feasibility of producing IgY from farm chickens immunized by recombinant Spike S1 protein subunit of SARS-CoV-2 Delta variant for potential diagnostics and therapeutics applications in pandemic emergencies.

Methods

This study was conducted in Mochtar Riady Institute for Nanotechnology (MRIN), Tangerang, Indonesia, for plasmid construction, cell culture, immunogen production, and protein analyses; and PT. IPB Shigeta Animal Pharmaceuticals, Bogor, Indonesia, for immunization of layer chicken and egg collection. The procedure of the IgY production is illustrated in Figure 1.

Plasmid Culture and Mutagenesis

A plasmid, pUNO1-SARS-S1-His-D614 (Cat. No. p1his-cov2-s1; InvivoGen, San Diego, CA, USA), designed for protein expression in mammalian cells and contained Wuhan-1 (D614) SARS-CoV-2 S1 subunit was inserted with an optimized signal sequence and codon usage. The plasmid was propagated in competent *Escherichia coli* DH5α (Cat. No. 18265017; Invitrogen, Darmstadt, Germany), purified using QIAamp Plasmid MidiPrep Kit (Cat. No. 12143; QIAGEN, Hilden, Germany), and the size was verified by Polymerase Chain Reaction (PCR) using GoTaq® PCR Core System I (Cat. No. M7660; Promega, Madison, WI USA). The pUNO1-SARS-S1-His-D614 plasmid was then modified by site-directed mutagenesis QuikChange II XL Site-Directed

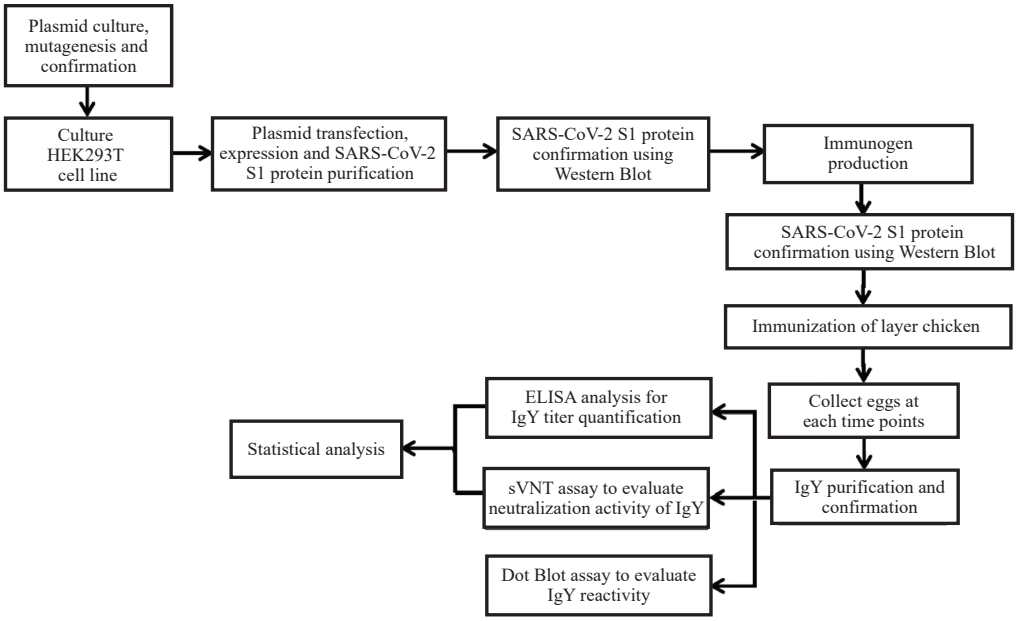


Figure 1. Flowchart of IgY production procedure.

Mutagenesis (Cat. No. 200522; Agilent, Saint Clara, USA) according to the manufacturer's protocol to generate S1 subunit Delta variant that possessed glycine at position 614 (614G), arginine at position 452 (452R) and glutamine at position 484 (484Q) amino acid residues, to create pUNO1-SARS-S1-His-D614G/L452R/E484Q. Mutation primers were as follow: for D614G mutation: S1-pUNO-mut D614G (F) TCCTGTATCAGGgCGTGAATTGCACCG and S1-pUNO-mut D614G (R) TCGGTGCAATTCACGcCCTGATACAGG; for L452R mutation: S1-pUNO-mut L452R (F) TGGCAACTATA ACTACCgGTATAGACTGTTCAGG and S1-pUNO-mut L452R (R) TCCTGAACAGTCTATACcGGTAGT TATAGTTGCC; for E484Q mutation: S1-pUNO-mut E484Q (F) TGCAACGGCGTGcAAGGGTTCAACTGC and S1-pUNO-mut E484Q (R) AGTTGAACCCTTgCACGCCGTTGCAGG. Successful mutagenesis was confirmed via Sanger method (Supplementary 1).

Cell Culture HEK293T

The human embryonic kidney containing SV40 large antigen (HEK293T) cells used were RIKEN (RCB2202) (RIKEN, Bioresource Center Cell Bank, Ibaraki, Japan), under a Material Transfer Agreement (MTA), and were exclusively cultured in the MRIN laboratory. HEK293T cells were grown in low-glucose Dulbecco's modified essential medium (DMEM) with pyruvate (Gibco, Waltham, MA, USA), supplemented with 10% FBS (Gibco) and Penicillin-Streptomycin (Gibco) in 5% CO_2 at 37°C.

Plasmid Transfection, Expression and Confirmation of SARS-CoV-2 S1 Subunit Protein

Protein expression was performed by transiently transfecting HEK293T cells (70–90% confluence) in a 6-well plate with 2.5 µg of pUNO1-SARS-S1-His-D614G/L452R/E484Q plasmid using Lipofectamine™ 3000 (Cat. No. L30000008; Invitrogen) in 1 mL medium according to the manufacturer's instructions. Different Lipofectamine amounts (1.5 or 3 µL/µg plasmid) were tested for optimal transfection efficiency, incubated at 37°C, 5% CO_2. Culture media were collected on day-3, -5, and -7 to determine the best incubation time. Mock cells received transfection reagent without plasmid. The culture media were concentrated using Amicon® Ultracel 10K (Millipore, Tullagree, Ireland) by centrifugation at 4000 x g, 4°C, for 15 minutes. Protein presence was measured by the Bradford method (Bio-Rad, Hercules, CA, USA) and confirmed by western blot.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% pre-cast acrylamide separating gel with the Precision Plus Protein Dual Color marker (Bio-Rad) and ran at 120 volts for 70 mins. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using Trans-Blot® TurboTM (Bio-Rad). The membrane was blocked with Intercept (TBS) Blocking Buffer (LI-COR Bioscience, Lincoln, NE, USA) and probed with mouse IgG anti-SARS-CoV-2 Spike S1 monoclonal antibodies (R&D System, Minneapolis, MN, USA) at 1:250. Detection was performed using IRDye 800CW goat anti-mouse secondary antibody (LI-COR Bioscience) diluted 1:10,000 in blocking buffer. The

membrane was scanned by Odyssey CLx Imager Instrument (LI-COR Bioscience).

Immunogen Production

For SARS-CoV-2-S1-Delta protein production, HEK293T cells grown in T-150 flasks were transfected with 37.5 µg pUNO1-SARS-S1-His-D614G/L452R/E484Q plasmid using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA) at 3 µL/µg plasmid in 15 mL medium. Cells were maintained in DMEM without FBS with Blasticidin (InvivoGen) at 30 µg/mL medium. The cells were stably secreting protein after being preserved in liquid nitrogen and recultured in DMEM with FBS and blasticidin. Protein was concentrated by using Amicon® Ultra-15 Centrifugal Filter (Millipore) and reconstituted in phosphate-buffered saline (PBS) (Life Technologies). The confirmation of SARS-CoV-2 S1 subunit was performed using western blot as formerly mentioned.

Immunization of Layer Chicken

SARS-CoV-2-S1-Delta reconstituted in PBS was mixed under high-shear conditions with Imject Freund's Incomplete Adjuvant (Thermo Fisher, Waltham, MA, USA) at a 1:1 v/v to achieve 1 mg protein/mL emulsion. Two pre-production six-week-old Hi-Brown layer hens (Ck-1 and Ck-2) were acclimated for 2 weeks before immunization in a protected cage with *ad libitum* water and a commercial diet. Immunizations were administered intramuscularly in the thigh on day-0, +21, +35, +56, and +77 at 1 mL per hen, containing 1 mg protein. Eggs were collected at multiple time points, *i.e*., on day of pre-immunization egg (-1), +21, +35, +50, +56, +71, +78, +92, +106 and +120. Hens were humanely euthanized via complete decapitation using sharp shears in a cutting cone. All animal procedures were approved by the Professor Nidom Foundation (PNF) Institution for Animal Care and Use Committee (IACUC) under the protocol No. 010620/IACUC/VII/2020.

IgY Purification

IgY purification was performed using the Pierce™ Chicken IgY Purification Kit (Cat. No. 44918; Pierce Biotechnology, Rockford, IL, USA) with 90% purity performance per egg yolk. The yolk was separated from the albumin, rolled over filter paper and mixed with a delipidating reagent at 5:1 ratio. After incubation at 4°C for 2 hours, centrifugation (4000 ×g, 4°C, 15 minutes) was performed. The supernatant was collected and IgY was precipitated by adding an equal volume of precipitation reagent, incubating at 4°C for 1 hour, followed by centrifugation at 4000 ×g, 4°C for 15 minutes. The pellet was resuspended in PBS and sterilized using a 0.45 µm filter. Based on the manufacturer's instruction, total IgY concentration was measured using NanoDrop 1000 (Thermo Scientific) using the provided IgY standard. SDS-PAGE confirmed IgY heavy and light chain at 67-70 kDa and 25 kDa, respectively.(21,26) A 12% pre-cast acrylamide gel was run at 150V for 55 mins using Precision Plus Protein All Blue (Bio-Rad) as a marker. The gel was stained using Coomassie Brilliant Blue for 1 hour, de-stained overnight and visualized.

Enzyme-linked Immunosorbent Assay (ELISA)

Commercial SARS-CoV-2 spike protein (InvivoGen) was diluted to 0.02 mg/mL in 0.05 M carbonate buffer (pH 9.6 to 9.7), and a total of 50 µL antigen was used to coat immunoassay plate wells (Nunc, Roskilde, Denmark). Blank wells contained only carbonate buffer, negative control wells were prepared without capture antibody, while positive control wells contained IgY anti-SARS-CoV-2 spike protein (My BioSource, San Diego, CA, USA). Plates were incubated at 37°C for 3 hours.

After incubation, antigen was discarded and wells were washed three times by PBS-Tween20, then blocked with BSA (Sigma-Aldrich, Waltham, CA, USA) at 37°C. IgY titration was performed by adding 100 µL IgY to the wells, with a serial dilution of IgY anti-SARS-CoV-2 spike protein (My BioSource) as standard and PBS-Tween20 as a negative control. The plate was incubated at 37°C, washed, then incubated with rabbit polyclonal antibody anti-chicken IgY-HRP (Abcam, Cambridge, MA, USA) diluted 1:10,000 in PBS-Tween20. After rewashing, enzymatic activity was detected by adding 100 µL TMB substrate and incubating for 10 minutes in the dark. The reaction was stopped by adding 1 M phosphoric acid, and optical density was measured at 450 nm using a DTX-880 Multimode Detector (Beckman Coulter, Brea, CA, USA).

Dot Blot Assay

A dot blot assay was performed on 10 SARS-CoV-2 positive nasopharyngeal swab lysate samples from routine clinical diagnostic testing for SARS-CoV-2 Spike using electrochemiluminescent immunoassay (ECLIA) Roche Elecsys SARS-CoV-2 Antigen ECLIA (Roche, Basel, Switzerland). Nitrocellulose membranes were spotted with 2 µL of samples and air-dried. Membranes were blocked using 5% BSA in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween20, pH 7.5), then incubated at room temperature with an in-house IgY antibody produced in this study (1:1,000 in TBS-T). Following incubation, the membranes were washed

three times with TBS-T. For detection, the membranes were incubated with a rabbit polyclonal anti-chicken IgY-HRP (diluted 1:10,000 in 5% skim milk in TBS-T) at room temperature and washed. Chemiluminescence detection was performed using WesternBright™ Quantum (Advansta, San Jose, CA, USA), and images were captured using a VersaDoc imaging system (Bio-Rad).

Surrogate Virus Neutralization Test (sVNT) Assay

The neutralization activity of IgY anti-S1 was demonstrated using sVNT (GenScript, Piscataway, NJ, USA). The SARS-CoV-2 sVNT kit was a blocking ELISA that mimics the virus neutralization process by evaluating the inhibition of horse radish peroxidase-conjugated receptor binding domain (HRP-RBD) binding to human ACE2 receptors by anti-SARS-CoV-2 RBD antibodies. Purified IgY anti-S1 (concentrated tenfold using Amicon® Ultra-15 Centrifugal Filter with a 100 kD cut-off), the positive control, or PBS (negative control), were mixed with HRP-RBD solution at a 1:1 v/v and incubated at 37°C for 30 minutes. Then, 100 µL of each mixture was added to the corresponding well of the ELISA plate and incubated at 37°C for 15 minutes. Following incubation, the plate was washed and 100 µL TMB solution was added. The plate was then incubated in the dark at room temperature for 15 minutes. The reaction was stopped by adding 50 µL stop solution and absorbance was measured at 450 nm using a DTX-880 Multimode Detector (Beckman Coulter). The inhibition rate was calculated using the following equation: Inhibition (%) = 100 x (1 - (OD of sample / OD of negative control)).

Statistical Analysis

The IgY anti-S1 ELISA and sVNT titers were analyzed using a two-tailed unpaired Student's t-test to compare the immunized groups with the pre-immunization titers. The $p<0.05$ were considered statistically significant.

Results

Plasmid Mutagenesis and Protein Expression

The original pUNO1His-SARS2-S1 plasmid contained the Spike S1 subunit coding sequences of the SARS-CoV-2 Wuhan-Hu-1 (accession no NC_045512), featuring aspartic acid residue at position 614 (D614), blasticidin-resistance gene (bsr), with an optimized signal sequence and codon-usage. Site-directed mutagenesis was performed on the pUNO1His-SARS2-S1 plasmid using QuikChange II XL Site-Directed Mutagenesis to introduce mutations corresponding to SARS-CoV-2 Delta variant as detailed in Table 1. The mutations confirmed by Sanger sequencing were presented in Supplementary 1.

Culture media from transfected and non-transfected (negative control) HEK293T cells in 6-well plates were collected at each time point and concentrated to ~200 µL to confirm S1 subunit protein expression. As shown in Figure 2A, a single band ~150 kDa was detected in all transfected samples but absent in the negative control. The strongest band appeared on day-3, suggesting this as the optimal incubation time for S1 subunit protein production.

Immunogen production was performed by similar transfection method, in T-150 cm^2 flasks, yielding a concentration ranging from 0.5-1 mg/mL medium. Culture media were collected and concentrated to ~1 mg/mL for confirmation. A SARS-CoV-2 Spike S1 protein fused to a poly-histidine tag (~123 kDa) served as the positive control. As shown in Figure 2B, both the immunogen and positive control displayed a single band at ~150 kDa, corresponding to the expected protein size.

High-Titer Neutralizing Anti-S1 IgY Produced from Immunized Layer Chicken

To evaluate the immune response induced by immunization, eggs were collected daily post-immunization to evaluate anti-S1-specific IgY titers, with pre-immunization eggs (day-1) serving as the baseline. IgY was purified from egg yolks and the purity was confirmed using SDS-PAGE. As shown in Figure 3A, the gel revealed distinct bands corresponding to the IgY heavy chain (~65 kDa), light chain (~25 kDa), and a truncated form of the heavy chain, confirming successful IgY purification. IgY titer was then quantified using ELISA, while neutralizing activity was evaluated by sVNT.

As shown in Figure 3B, both chickens (Ck1 and Ck-2) demonstrated increased anti-IgY titers following the second immunization. Ck-2 consistently showed higher IgY peaking at 2.08 mg/mL on day-56, while Ck-1 reached a maximum

Table 1. Site-directed mutagenesis of pUNO1His-SARS2-S1 for SARS-CoV-2 Alpha and Delta variants.

Codon-optimized	614		452		484	
	N	A	N	A	N	A
pUNO1His-SARS2-S1 (Wuhan-Hu-1)	GAC	D	CTG	L	GAA	E
pUNO1His-SARS2-S1 D614G/L452R/E484Q (Delta variant)	GGC	G	CGG	R	CAA	Q

N: nucleotide, A: amino acid.

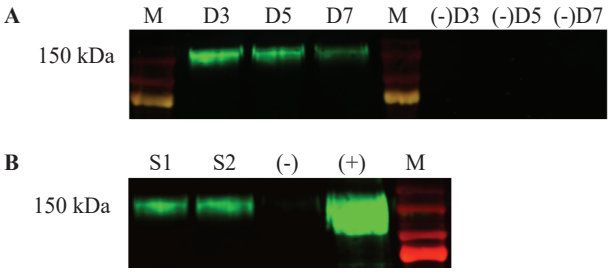


Figure 2. Confirmation of SARS-CoV-2 S1 subunit protein expression. A: Optimization of transfection duration in HEK293T cells using three incubation times resulted three days incubation was the most optimal incubation time. M: Marker; D3, D5, D7: media collected on day-3, 5, and 7 post-transfection; (-)D3, (-)D5, (-)D7: non-transfected controls collected on the corresponding days. B: Reproducibility of S1 subunit protein expression from day-3 transfection, confirming its suitability for immunogen production. S1: S1 transfected sample; S2: S2 transfected sample; (-): negative control; (+): positive control; M: Marker.

of 1.59 mg/mL on day-120. Statistical analysis of the mean of IgY titers when compared to the pre-immunization titer (Figure 3C) showed significantly elevated IgY titers on day-92, -106, and -120, or after the fifth immunization, *i.e.*, 1.21, 1.72 and 1.48 (p=0.0076, 0.0399 and 0.0062), respectively, confirming a robust antibody response over time.

Neutralizing activity, as measured by via surrogate virus neutralization test (% sVNT), also increased over time, with significant elevations observed beginning at day 50. Based on manufacturer's manual, sVNT titer cut-off to be considered had the neutralizing potency was >20%. sVNT results mirrored the anti-IgY titer trends, with Ck-2 achieving a maximum neutralization of 51.3%, and Ck-1 reaching 62.8% by day 120. The mean of sVNT titers when compared to pre-immunization titer was consistently significant after the third immunization, *i.e.*, day 50, 56, 78, 92, 106 and 120, *i.e.*, 31.9%, 34.95%, 26.9%, 47.6%, 54.95% and 57.3% (p=0.0005, 0.0021, 0.0324, 0.0280, 0.0004 and 0.0094), respectively (Figure 3C).

A slight decline in IgY titers was observed in both chickens after the fourth immunization (day-71), which was followed by a marked increase after the fifth dose, especially in Ck-1. This pattern highlights the importance of booster immunizations for maintaining antibody levels.

Increase of Specific IgY Proportion Relative to Total IgY Following Immunization

To assess the yield of specific IgY against the S1 subunit, we compared anti-S1 IgY to total IgY levels at each time point, as shown in Figure 3D. Relative anti-S1/total IgY ratios also increased significantly in both chickens, with Ck-2 exhibiting higher overall specificity. On average, the proportion of anti-S1 IgY reached 5% three weeks after the first immunization, though this was observed in only one hen. By day-35 (two weeks after the second immunization), measurable anti-S1 IgY was detected in both hens, accounting for 17% of total IgY. The proportion of specific IgY continued to rise, peaking at 29% after the fifth immunization on day-106 (p<0.05). These results indicate a progressive increase in S1-specific IgY following repeated boosters, though responses varied between individuals.

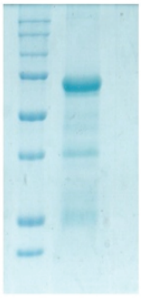
Reactivity of Anti-S1 IgY Antibodies Against Clinical Samples

There was limited information on whether SARS-CoV-2 specific IgY could bind to clinical specimens. In this study, we investigated the reactivity of anti-S1 IgY to nasopharyngeal swab samples collected from confirmed COVID-19 patients. The IgY sample used was collected from Ck-2 on day-106, corresponding to the highest anti-S1 IgY yield following the final immunization. Clinical specimens were leftover nasopharyngeal swabs that had been lysed and tested positive using the ECLIA for SARS-CoV-2. After testing, the lysates were stored at -4°C and subsequently used in a dot blot assay within 72 hours of collection to assess IgY reactivity. As shown in Figure 4, all clinical specimens produced visible gray spots on the membrane, indicating positive binding with the anti-S1 IgY. These results demonstrate that IgY antibodies generated in immunized chickens can recognize SARS-CoV-2 antigens in actual clinical samples.

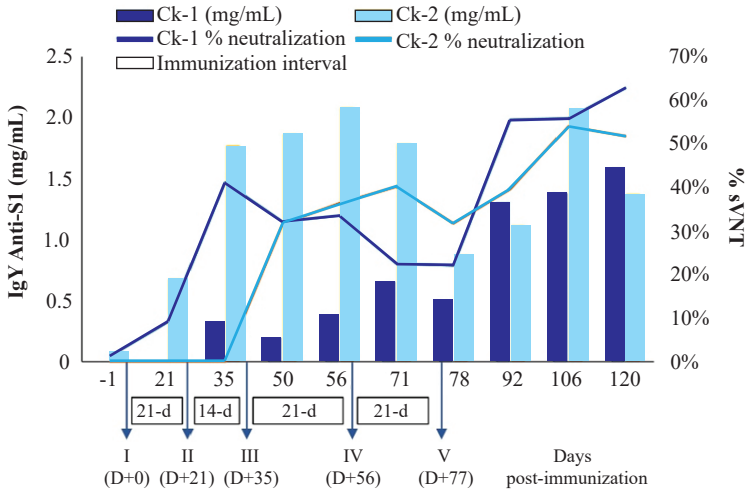
Discussion

The potential of IgY for the prophylaxis of infectious diseases, including the evolving COVID-19 pandemic, has been previously reported.(23,26,29) Although the ideal production of IgY is conducted using specific-pathogen-free (SPF) chickens, SPF facilities are costly, limited in availability, and generally not equipped for large-scale production.(12,30) Therefore, layer chicken farms serve as practical alternative. Moreover, scaling IgY production at local farm conditions is remarkably cost-effective, due to the relatively low-cost maintenance compared to the substantial overhead associated with laboratory facilities. (22) Initially, this study aimed to produce IgY using SARS-CoV-2 field isolates as the immunogen in a group of twenty hens. However, at the time, no institution could provide a continuous, large-scale supply of SARS-CoV-2

A



B



C

		Duration Post-immunization (days)									
		-1	**21**	**35**	**50**	**56**	**71**	**78**	**92**	**106**	**120**
Ck-1	IgY anti-S1 (mg/mL)	0.00	0.00	0.30	0.19	0.38	0.65	0.50	1.30	1.38	1.59
	% sVNT	1.3	9.0	41.2	32.2	33.5	22.5	22.1	55.6	55.9	62.8
Ck-1	IgY anti-S1 (mg/mL)	0.07	0.67	1.77	1.86	2.08	1.78	0.87	1.11	2.07	1.37
	% sVNT	0	0	0	31.6	36.4	40.2	31.7	39.6	54.0	51.8
	Means of IgY anti-S1 (mg/mL)	0.04	0.34	1.03	1.03	1.23	1.22	0.69	1.21	1.72	1.48
	p-value of IgY anti S1		0.4671	0.3076	0.3567	0.2970	0.1714	0.0744	0.0076*	0.0399*	0.0062*
	Means of % sVNT	0.6	4.5	20.6	31.9	34.9	31.3	26.9	47.6	54.9	57.3
	p-value of % sVNT		0.4863	0.4352	0.0005*	0.0021*	0.0744	0.0324*	0.0280*	0.0004*	0.0094

D

		Duration Post-immunization (days)									
		-1	**21**	**35**	**50**	**56**	**71**	**78**	**92**	**106**	**120**
Ck-1	Relative anti-S1/total IgY	0.000	0.000	0.062	0.034	0.063	0.111	0.090	0.212	0.227	0.216
Ck-2	Relative anti-S1/total IgY	0.011	0.100	0.277	0.282	0.335	0.302	0.143	0.199	0.358	0.246
	Means of anti-S1/total IgY (%)	1	5	17	16	20	21	12	21	29	23
	p-value of anti-S1/total IgY		0.4716	0.2686	0.3448	0.2926	0.1696	0.0557	0.0019*	0.0488*	0.0050*

Figure 3. Anti-S1 IgY production and neutralizing activity in immunized layer chickens. A: SDS-PAGE analysis of purified IgY showing distinct bands corresponding to the heavy chain, truncated heavy chain, and light chain. B: ELISA quantification of anti-S1 IgY titers in milligram in each milliliter of total IgY (primary Y axis, bars) and percentage of virus neutralization measured by surrogate virus neutralization test (sVNT) (secondary Y axis, lines) over time in two chickens (Ck-1 and Ck-2). Ck-1: blue bars (IgY titer) and blue line (% sVNT); Ck-2: orange bars (IgY titer) and orange line (% sVNT). Arrows indicate immunization time points with 2–3 weeks intervals. C: Quantitative comparison of IgY titers and sVNT percentages for each chicken at specified time points, including statistical significance (*p<0.05). D: Relative anti-S1 IgY to total IgY levels, indicating increasing specificity post-immunization, with significant differences highlighted (*p<0.05).

viral cultures, thus we could not conduct the study in a larger group of animals. Given these constraints, this study was re-designed with three main objectives: to produce recombinant SARS-CoV-2 Spike S1 subunit protein during COVID-19 pandemic emergency as alternative to field isolate; to generate anti-S1 IgY in farm layer chickens using the recombinant protein as the immunogen; and to assess the potency of the resulting IgY based on specific antibody titer, neutralizing activity, and reactivity to clinical specimens.

The sVNT is widely used to evaluate the potency of neutralizing antibodies without the need for live virus or biosafety level 3 (BSL-3) facilities.(31,32) Due to the limited time and our laboratory-scale recombinant protein production, we were only able to produce enough immunogen for two hens at each time point. This has been a major limitation of this study. However, the use of recombinant S1 protein produced in-house successfully elicited strong immune responses in chickens, with a dose of 1 mg per injection in 2-3 weeks interval. While other studies report effective doses ranging from 2.5 to 500 µg, (12,30,33,34) we did not encounter any difficulty producing sufficient quantities of recombinant protein. For non-SPF

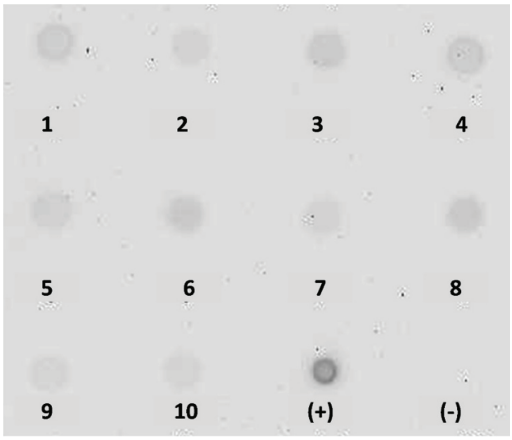


Figure 4. Reactivity of IgY anti-S1 from Ck-1 against clinical specimens. Samples 1–10 represent leftover lysates from nasopharyngeal swabs of ten patients who tested positive for SARS-CoV-2 using the Elecsys Roche CLIA rapid SARS-CoV-2 assay. (+): SARS-CoV-2 S1 control protein; (–): sample diluent (negative control).

chickens, we considered hyperimmunization at 1 mg/injection to be more effective. Nonetheless, we recognize that dose optimization is important for future cost-efficient production. The recombinant S1 protein used in this study induced a strong IgY response, with specific anti-S1 IgY comprising 17% of total IgY after the second immunization and increasing to 29% after the fifth. Previous studies have reported that only 2–10% of total IgY in immunized farm hens is specific to the target antigen (35), suggesting that the IgY response we achieved is comparatively high. This indicates that the recombinant S1 subunit protein from the Delta variant can induce a substantial yield of neutralizing IgY in farm chickens. Importantly, the anti-S1 IgY produced in this study demonstrated reactivity against clinical specimens collected during a period when the Omicron variant accounted for over 90% of SARS-CoV-2 infections. (1,36) This suggests that the generated antibodies may offer cross-reactivity with current circulating variants. However, a major limitation is that the clinical samples were residual lysates from point-of-care testing and were not sequenced to confirm the infecting variant. Moreover, the number of specimens was limited.

According to the 2019 technical guidance from the Indonesian Ministry of Agriculture, farm layer chickens routinely receive vaccinations against various diseases, including Newcastle disease, avian influenza, infectious bursal disease, Coryza, Marek's disease, and infectious bronchitis, from one month of age.(37) This suggests that chickens raised under standard commercial vaccination programs are still capable of mounting robust immune responses to novel antigens like recombinant S1, although SPF chickens remain the gold standard for IgY production. Even though in this study, the number of chickens used (n=2) was small, the immunization dose optimization was not performed, and there was no challenge test was conducted; yet this study offered a feasible and rapid-response strategy during the COVID-19 emergency. Although SPF chickens may give a higher yield and less individual variety, farm layer chickens provide a practical platform in terms of infrastructure, nationwide availability, and cost-effectiveness. Although we had the expertise to conduct additional experiments, constraints during the study period, including lack of animal BSL-3 facilities, unavailability of local virus isolates, limited emergency funding, and tight timelines, prevented us from performing further validation. Future studies should include a larger number of animal replicates, challenge tests in animal and virus neutralization assays using circulating field isolates, and a larger number of clinical isolates for functionality tests to confirm and validate the efficacy of the IgY antibodies produced.

Conclusion

A high-yield and functional IgY anti-S1 SARS-CoV-2 Delta variant can be produced from farm chicken immunized with an in-house recombinant protein. This study offers a proof-of-concept of a safe, scalable, and cost-effective method to produce functional anti-Spike IgY antibodies for diagnostic or treatment in farm chickens during pandemic emergencies in low-resource settings.

Acknowledgments

We thank dr. David Rustandi (Siloam Hospitals, Lippo Village) for providing clinical specimens and Mochtar Riady Institute for Nanotechnology for supporting research facilities. This research is funded through the Indonesia Research and Innovation Funding Program for Coronavirus Disease 2019 (COVID-19) No. 11/F1/PKS-KCOVID-19.D/VII/2020, No. 001/CVD-IPBSGT/VII/20.

Authors Contribution

KZ was the conceptor and principal investigator. KZ, TEG and AHP designed the experiment. FA, ACL, and AN collected the study data. BR designed ELISA protocol,

while TEG performed data analysis. TEG and VC wrote the original manuscript draft. AHP and IY discussed result and reviewed the manuscript. All authors have approved the final article.

Conflict of Interest

The authors declare no conflicts of interest or competing interests related to the content of this manuscript.

References

1. World Health Organization [Internet]. Weekly epidemiological update on COVID-19 [updated 2022 Mar 22; cited 2024 Jan 10]. Available from: https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19---22-march-2022.
2. Lestari K, Babikian H, Kulsum ID, Ferdian F, Ismail E, Sumalim Y, *et al.* The combination of Gardenia jasminoides, Boswellia serrata, Commiphora myrrha, Foeniculum vulgarae, and Daucus carota essential oil blend improved the inflammatory and clinical status in respiratory tract infection of COVID-19 patients: A multicentre, randomized, open-label, controlled Trial. Indones Biomed J. 2024; 16(3): 237–47.
3. Chukkayapalli SG, Suravaram S, Reddy BK, Siddiqui IA. Genotype AA of ACE2 G8790A (rs2285666) has protective potential against COVID-19 disease severity. Mol Cell Biomed Sci. 2023; 7(3): 155–61.
4 Centers for Disease Control and Prevention [Internet]. Symptoms of COVID-19 [cited 2024 Jan 10]. Available from: https://www.cdc.gov/coronavirus/2019-ncov/symptoms-testing/symptoms.html
5. Tandirogang N, Fitriany E, Mardania N, Jannah M, Dilan BFN, Ratri SR, *et al.* Neutralizing antibody response by inactivated SARS-CoV-2 vaccine on healthcare workers. Mol Cell Biomed Sci. 2023; 7(1): 18–27.
6. Shanmugaraj B, Malla A, Phoolcharoen W. Emergence of novel coronavirus 2019-nCoV: Need for rapid vaccine and biologics development. Pathogens. 2019; 9(2): 148. doi: 10.3390/pathogens9020148.
7. Hoffmann M, Kleine-Weber H, Pohlmann S. A multibasic cleavage site in the spike protein of SARS-CoV-2 is essential for infection of human lung cells. Mol Cell. 2020; 78(4): 779–84.
8. Shang J, Wan Y, Luo C, Ye G, Geng Q, Auerbach A, *et al.* Cell entry mechanisms of SARS-CoV-2. Proc Natl Acad Sci. 2020; 117(21): 11727–34.
9. Wilks SH, Mühlemann B, Shen X, Türeli S, LeGresley EB, Netzl A, *et al.* Mapping SARS-CoV-2 antigenic relationships and serological responses. Science. 2023; 382(6666): eadj0070. doi: 10.1126/science.adj0070.
10. Premkumar L, Segovia-Chumbez B, Jadi R, Martinez DR, Raut R, Markmann AJ. The receptor-binding of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. Sci Immunol. 2020; 5(48): eabc8413. doi: 10.1126/sciimmunol.abc8413.
11. Yuan M, Liu H, Wu NC, Wilson IA. Recognition of the SARS-CoV-2 receptor binding domain by neutralizing antibodies. Biochem Biophys Res Commun. 2021; 538: 192–203.
12. Lu Y, Wang Y, Zhang Z, Huang J, Yao M, Huang G, et al. Generation of Chicken IgY against SARS-COV-2 Spike Protein and Epitope Mapping. J Immunol Res. 2020; 2020: 9465398. doi: 10.1155/2020/9465398..
13. Callaway E. Delta coronavirus variant: Scientists brace for impact. Nature. 2021; 595(7865): 17–8.
14. GSAID [Internet]. Tracking of hCoV-19 Variants [cited 2025 May 13]. Available from: https://gisaid.org/hcov19-variants/.
15. Latarissa IR, Barliana MI, Meiliana A, Sormin IP, Sugiono E, Kartasasmita CB, *et al.* Efficacy of quinine sulfate in patients with mild-to-moderate COVID-19: A randomized controlled trial. Indones Biomed J. 2023; 15(6): 366–74.
16. Lu H. Drug treatment options for the 2019-new coronavirus (2019-nCoV). Biosci Trends. 2020; 14(1): 69–71.
17. Wang M. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res. 2020; 30(3): 269–71.
18. Sparrow E, Friede M, Sheikh M, Torvaldsen S. Therapeutic antibodies for infectious diseases. Bull World Heal Organ. 2017; 95(3): 235–7.
19. Graham BS, Ambrosino DM. History of passive antibody administration for prevention and treatment of infectious diseases. Curr Opin HIV AIDS. 2015; 10(3): 129–34.
20. Shen C, Wang Z, Zhao F, Yang Y, Li J, Yuan J, *et al.* Treatment of 5 critically ill patients with COVID 19 with convalescent plasma. JAMA. 2020; 323(16):1582–9.
21. Pereira EPV, van Tilburg MF, Florean EOPT, Guedes MIF. Egg yolk antibodies (IgY) and their applications in human and veterinary health: A review. Int Immunopharmacol. 2019; 73: 293–303.
22. Schade R, Calzado EG, Sarmiento R, Chacana PA, Porankiewicz-Asplund J, Terzolo HR. Chicken egg yolk antibodies (IgY-technology): A review of progress in production and use in research and human and veterinary medicine. Altern Lab Anim. 2005; 33(2): 129–54.
23. Rahman S, Van Nguyen S, Icatlo FC Jr, Umeda K, Kodama Y. Oral passive IgY-based immunotherapeutics: A novel solution for prevention and treatment of alimentary tract diseases. Hum Vaccin Immunother. 2013; 9(5): 1039–48.
24. Krief A, Letesson J, Billen D. Comparison between 'IgY technology' from chickens and 'IgG technology' from mice for production of tailor-made antibodies. Tetrahedron Lett. 2020; 43(10): 1843–6.
25. Pérez de la Lastra JM, Baca-González V, Asensio-Calavia P, González-Acosta S, Morales-delaNuez A. Can immunization of hens provide oral-based therapeutics against COVID-19? Vaccines. 2020; 8(3): 486. doi: 10.3390/vaccines8030486.
26. Lee L, Samardzic K, Wallach M, Frumkin LR, Mochly-Rosen D. Immunoglobulin Y for potential diagnostic and therapeutic applications in infectious diseases. Front Immunol. 2021; 12: 696003. doi: 10.3389/fimmu.2021.696003.
27. Vega CG, Bok M, Vlasova AN, Chattha KS, Fernández FM, Wigdorovitz A, *et al.* IgY antibodies protect against human rotavirus induced diarrhea in the neonatal gnotobiotic piglet disease model. PLoS One. 2012; 7(8): e42788. doi: 10.1371/journal.pone.0042788.
28. Torché A-M, Dimna M Le, Corre P Le, Mesplède A, Gal S Le, Cariolet R, *et al.* Immune responses after local administration of IgY loaded-PLGA microspheres in gut-associated lymphoid tissue in pigs. Vet Immunol Immunopathol. 2006; 109(3–4): 209–17.
29. Abbas AT, El-Kafrawy SA, Sohrab SS, Azhar EIA. IgY antibodies for the immunoprophylaxis and therapy of respiratory infections. Hum Vaccin Immunother. 2019; 15(1): 264–75.
30. Bao L, Zhang C, Lyu J, Yi P, Shen X, Tang B, *et al.* Egg yolk immunoglobulin (IgY) targeting SARS-CoV-2 S1 as potential virus entry blocker. J Appl Microbiol. 2022; 132(3): 2421–30.

31. Rhein C, Scholz T, Henss L, Wiedemann R, Scwarz T, Rodionov R, *et al.* Comparison of potency assays to assess SARS-CoV-2 neutralizing antibody capacity in COVID-19 convalescent plasma. J Virol Methods. 2021; 288: 114031. doi: 10.1016/j.jviromet.2020.114031.
32. Armimi A, Syuaib AF, Vanya K, Tan MI, Natalia D, Chen DV, *et al.* SARS-CoV-2 neutralization assay system using pseudo-lentivirus. Indones Biomed J. 2023; 15(2): 179–84.
33. Aston EJ, Wallach MG, Narayanan A, Egaña-Labrin S, Gallardo RA. Hyperimmunized chickens produce neutralizing antibodies against SARS-CoV-2. Viruses. 2022; 14(7):1510. doi: 10.3390/v14071510.
34. Ravlo E, Evensen L, Sanson G, Hildonen S, Ianevski A, Skjervold PO, *et al.* Antiviral immunoglobulins of chicken egg yolk for potential prevention of SARS-CoV-2 infection. Viruses. 2022; 14(10): 2121. doi: 10.3390/v14102121.
35. Schade R, Bürger W, Schöneberg T, Schniering A, Schwarzkopf C, Hlinak A, *et al.* Avian egg yolk antibodies. The egg laying capacity of hens following immunisation with antigens of different kind and origin and the efficiency of egg yolk antibodies in comparison to mammalian antibodies. ALTEX. 1994; 11(2): 75–84.
36. Institute for Health Metrics and Evaluation [Internet]. COVID-19 Results Briefing [updated 2022 Dec 15; cited 2024 Jan 10]. Available from: https://www.healthdata.org/sites/default/files/covid_briefs/15_briefing_Myanmar.pdf.
37. Wiyono A, Adjid RMA, Suhardono, Widiastuti R, Noor SM, Nuradji H, *et al*. Petunjuk teknis aspek kesehatan hewan ternak ayam mendukung program perbibitan ternak balitbangtan dan program bedah kemiskinan rakyat sejahtera (“bekerja”) di Balitbangtan. Bogor: Balai Besar Penelitian Veteriner; 2019.